INSTRUCTIONS FOR COMPLETING THE NIH INTRAMURAL ANIMAL STUDY PROPOSAL FORM

The Animal Welfare Act (AWA) regulations and the Public Health Service Policy on the Humane Care and Use of Laboratory Animals (PHS Policy) require review and approval of an Animal Study Proposal (ASP) by the Animal Care and Use Committee (ACUC) prior to the initiation of any research activity involving vertebrate animals.

GENERAL INSTRUCTIONS:

These instructions are intended as an aid for the completion of the ASP form. The information that you provide on this form should represent a plan for the research to be conducted. It will be evaluated to assure that the research proposed is an appropriate use of the animals, and that technical assistance and appropriate facilities are available to support the research. NIH considers all information submitted in the ASP to be privileged and confidential. Once the ASP is approved, however, portions of the ASP that are not exempt from disclosure under the Freedom of Information Act (FOIA) are subject to release if a request is made under the FOIA. NIH would also withhold any confidential information contained in the ASP that is exempt from disclosure under the FOIA. If you have questions about the information that is requested on any part of the ASP form, call your IC Veterinarian's office, or the Chairperson of your ACUC.

Reference documents: All regulatory and guidance documents referred to in these instructions are provided in *Using Animals in Intramural Research: Guidelines,* behind the “Laws, Regulations & Policies” tab. This notebook is given to all persons who attend the training courses for Principal Investigators and Animal Users.

SECTION A: ADMINISTRATIVE DATA

Principal Investigator: The ACUC realizes that there may be more than one investigator involved in a study but *one* person must assume responsibility for the ASP and its execution.

Bldg/Room, E-mail, Telephone, FAX: Provide complete and accurate information, as this will be utilized for the life of the ASP.

Submitted ASP: Indicate whether this is an initial submission, renewal or modification of an existing ASP. If this is not an initial submission, enter in the number of the approved proposal.

Key Personnel: List all personnel who will be conducting procedures involving animals under this ASP.

SECTION B: ANIMAL REQUIREMENTS

Species, Age/Weight/Size, Sex, Stock or Strain: Provide specific information regarding these characteristics of the animals. List all vendors, stocks, and strains that will be used. If you are using a transgenic or knock-out strain of mice, provide the background strain(s) and the designation of the genetic mutation.

Source: Rodents are generally ordered from approved vendors through the Veterinary Resources Program (VRP) centralized animal procurement service (CAPS). For more information about all aspects of the animal procurement process, contact your IC Veterinarian. If rodents or rodent products are obtained from a source other than the VRP list of "approved sources" a NIH Form 2369-1, *Application for Permit to Introduce Rodents and Rodent Products*, (See OACU notebook, tab VI), must be submitted through the IC Veterinarian.

Holding Location(s): Indicate where animals are proposed to be housed (building and room). **NOTE:** If it is necessary to hold animals anywhere outside of a core animal facility for more than 12 hours, you must contact your ACUC chairperson and identify the building and room number(s).

Animal Procedure Location(s): Provide the building and room number for all laboratory spaces, or animal facility spaces that will be used for animal procedures to include surgeries, euthanasia, and tissue culture work.

Number of Animals: Estimate the number of animals to be used each year of the approval period, and the total number of animals to be used (ASPs may be approved for a maximum of three years). If multiple species are used, provide these numbers for each species. If the study is a renewal, remember to account for animals to be carried over from the previous protocol.

SECTION C: TRANSPORTATION

Requirements for proper transportation of animals are described in the "NIH Animal Transportation Guidelines" (See OACU notebook, Tab VIII) to assure the security and safety of the animals and humans involved.

Transport of animals through the Clinical Center must comply with the "Research Animal Transportation Policy 614, (Building 10)". (See OACU notebook, Tab IX.)

If you are uncertain of the policies applicable to your study, additional information is available from your IC Veterinarian's office.

SECTION D: STUDY OBJECTIVES

In *non-technical* terms, describe the aim(s) of the study to include how the study may benefit human or animal health or advance scientific understanding of biological processes. This non-technical description is directed to the lay member of the ACUC to facilitate their understanding of the ASP.

SECTION E: RATIONALE FOR ANIMAL USE

1) Animal Model: Why is an animal model necessary for this study? Why can't cell culture, computer simulations or other non-animal models be used in place of the proposed animal model?

Examples:
This study depends on complex behavioral activities that require a functioning animal with a highly developed nervous system.

The investigation of the effects of anterior hypothalamic lesions on immune responses can only be performed on living organisms with well-developed and intact nervous and immune systems.

Living animal cells are necessary to study the translation of exogenous mRNAs. Oocytes have been used very successfully for these types of studies.

2) Appropriateness of the species selected: Describe the biological characteristics of the animal that are essential to the proposed study; provide a reason(s) that other species, particularly a lower order mammal, invertebrate, etc. are not suitable.

3) Number of animals:
Most studies at NIH are either new experimental paradigms or pilot studies, and therefore, applying statistical principles to justify numbers can be difficult due to the lack of knowledge regarding the potential outcomes and variance. Under these circumstances, it is still important to show how animal numbers were generated. It is also reasonable to ask for larger group sizes (i.e. 10/group versus 6/group) with the caveat that early statistical analysis will be applied and the group size decreased if the statistics support using smaller groups.

For Example: We estimate that 200 animals will be necessary for this study because we will be using five (5) animals per group, and examining the effects of five (5) compounds (including vehicle), at four (4) doses of drug per compound, with two (2) replications, (to assure reproducibility), per determination. Therefore the total numbers requested will be: 5 x 5 x 4 x 2 = 200.

In studies that are a continuation of ongoing work or that parallel current or previous experiments, if the degree of variance is known, then a short summary of statistical principles that were applied to arrive at your group size(s) is appropriate (i.e. power, standard deviations, etc.). If applying statistical principles is not directly applicable, then a short summary of reasoning applied to arrive at group sizes should be provided.

When the use of animals is for the harvesting of normal tissues, organs or fluids for in vitro use: Briefly cite expected usage levels to provide the quantity of tissues or fluids needed for the study. If no prior experience is available, state an anticipated tissue/fluid harvest per animal with a description of the process. These numbers should be scientifically justified and not based solely on personnel availability.

For Example: In our experience, 10 rats are required to generate enough cells for one experiment. Since we can conduct one experiment per week we need 520 rats per year. This number of animals and rate of use will enable us to test 10 drugs at the desired dosage.

If rodents are to be used for breeding, then the following categories can be used in justifying/clarifying your animal numbers: 1) numbers used for breeding (including founders, background strain, and retained progeny), 2) total numbers of expected progeny, 3) the numbers of progeny intended for experimental use or export, 4) the numbers of progeny needed for continuation of the experimental line, and 5) the numbers that will be euthanized due to undesirable genotype. Animals with undesirable genotype are not counted into animal total numbers if they are euthanized before weaning, but *for completeness* should be listed in the numerical breakdown. In addition, it is helpful to provide the objectives for the breeding (i.e. homozygous pups for experiments, or back crossing to establish a genetic mutant on a homogeneous background). Enough detail of the breeding schemes should be included to allow the ACUC to assess the appropriateness of requested animal numbers. An outline, a table, or a flow chart for each strain listed can be very helpful in presenting this information. This breakdown of breeding categories should be included under Section F as part of the experimental design and referenced in Section E.3. See **Attachment 1** for examples of commonly employed breeding schemes and instructions on estimating animal numbers for this purpose.

SECTION F: EXPERIMENTAL DESIGN AND ANIMAL PROCEDURES:

Describe the experimental design clearly. Include enough information in the description of the procedures to enable the ACUC members to visualize each procedure as it will be performed. Use the description of procedures on the form as a model for the level of detail desired. Include anticipated effects on the animals or personnel coming in contact with the animals. Provide specific details for each procedure that can affect the pain or distress potential for the animals.

Anesthetic Regimens: Describe in ASP SECTION I.

Hazardous Agents: Describe in SECTION K

Special Concerns: Describe in SECTION M

Endpoint Criteria: Provide an experimental endpoint such as a timeline that should be met or medical state that should be achieved (i.e. a 1 cm tumor) that will determine the time for euthanasia or withdrawal from the study. In studies where adverse outcomes or complications might be expected, then humane endpoints should also be described. For example, the pain experienced by an arthritic mouse mutant may be alleviated with analgesics, but lack of proper mobilization may ultimately cause body weight loss and a need for euthanasia prior to reaching the experimental endpoint. In this case, setting a body weight loss standard of 15-20% would constitute a humane endpoint. For all studies, death as an experimental endpoint should be minimized and must be scientifically justified. Please refer to the ARAC Guideline, “Endpoints in Animal Study Proposals” (3/8/00) for further details on setting appropriate endpoints for column D & E studies.

SECTION G: SURVIVAL SURGERY

DEFINITIONS:

Survival Surgery: A surgical procedure from which an animal is allowed to recover from general anesthesia.

Non-Survival Surgery: A surgical procedure in which the animal is euthanized prior to recovery from general anesthesia. (Describe under section F)

Major Operative Procedure: A procedure that penetrates a body cavity, or any procedure that has the potential for producing permanent impairment of physical or physiological function.

Vascular cutdowns and subcutaneous implants *are not* considered major operative procedures.

1) Surgical Procedure/Aseptic Technique: Survival surgery on rodents should be performed in accordance with the “NIH Intramural Research Program Guidelines for Survival Rodent Surgery”. (See OACU Notebook, Surgery/Postsurgical Care Tab)

Sterile instruments and aseptic technique are required for ALL species (rodents, rabbits, dogs, etc.). Describe patient prep, i.e. pre-operative medications and/or hair clipping and skin disinfection procedures. Describe intraoperative support procedures for the animal, i.e. methods for maintaining body temperature, and methods for assessing depth of anesthesia (heart rate, respiration rate, etc.). Describe methods of instrument sterilization for the initial surgery and (for rodents) between surgeries, i.e. cold sterilant, hot beads, etc.

2) Surgeon's Qualifications: Provide the names of all individuals performing animal surgery and describe their qualifications to perform the specific procedures listed in terms of related training and experience.

3) Location: Specify the Building and Room number where survival surgeries will be performed. Specialized surgical facilities are required for survival surgical procedures proposed in rabbits and higher species such as cats, dogs, and primates. Surgery on rodents must be performed in suitably prepared areas in accordance with "NIH Intramural Research Program Guidelines for Survival Rodent Surgery". (See OACU notebook, Surgery/Postsurgical Care Tab)

4) Post-Operative Care: Describe supportive therapy that is required (e.g. supplemental heat source, I.V. fluids, etc.) and state the observations the designated person will use to evaluate the animal’s health status.

5) Prior Surgery: Answer yes or no according to whether or not major surgery has been performed on any animal prior to its placement on this study. If so, explain the circumstances.

For Example: When surgically altered animals are obtained from a vendor, state the name of the procedure and the name of the vendor who performed it.

6) Multiple Procedures: Multiple major survival surgical procedures on a single animal must be scientifically justified and approved by the ACUC.

SECTION H: PAIN AND DISTRESS CATEGORY

Pain and Distress: Contact your IC Veterinarian for guidance in classifying procedures by the expected levels of pain or distress produced by the procedures. The U.S. Department of Agriculture (USDA) requires an annual report of the number of animals used in research which exposed them to one of three categories of pain or distress: C (minimal, transient or no pain or distress), D (pain or distress relieved by appropriate measures) or E (Unrelieved pain or distress). (For examples, see ATTACHMENT 2)

The animal numbers displayed in this section should match the total numbers listed in Section B. If multiple species are used, then list each species separately.

For animals categorized as column D or E, a written narrative must be provided describing the sources and methods used to consider alternatives to these procedures. The majority of researchers meet this requirement by performing 2 or more database searches such as MEDLINE, AGRICOLA or ALTWEB. When database searches are performed, you need to include the databases searched, the date of the search, the years covered by the search, and the keywords and /or search strategy used." Your IC veterinarians or the NIH library staff (301-594-6200) can provide information on appropriate key words and databases to use for your area of research.

In some highly specialized fields of study, conference proceedings, subject-expert consultants, etc., may provide more relevant and current information. If this method is used, sufficient documentation must be provided to demonstrate the viability of this information in addressing the issue of alternatives.

SECTION I: ANESTHESIA, ANALGESIA, TRANQUILIZATION

The type and dose of anesthetic, analgesic, and tranquilizer or sedative must be appropriate for both the species being used and the type of pain or distress being prevented/relieved. Doses and routes of administration should be clearly appropriate and effective, i.e., commonly accepted or published doses, or through a description of your experience with the agent and dose described which demonstrates its effectiveness.

The frequency and/or indications for drug administration should be provided, e.g. every 12 hours, as needed, etc. If agents are to be given "as needed", a brief description of the indications for its administration should be provided, e.g., "at the first indication of discomfort as evidenced by lethargy, anorexia, hunched posture, eye squinting, or vocalization."

SECTION J: METHOD OF EUTHANASIA

Methods which are not consistent with the recommendations in the 2000 Report of the AVMA Panel on Euthanasia must be scientifically justified in writing. (See OACU notebook, Euthanasia Tab)

Animal carcasses which have not been contaminated with hazardous agents are to be disposed of as Medical Pathological Waste in accordance with NIH Division of Safety guidelines.

Division of Safety personnel (496-2346) should be consulted on proper disposal methods for carcasses contaminated with hazardous agents.

SECTION K: HAZARDOUS AGENTS

The use of hazardous agents requires the concurrence of the appropriate NIH OHSB safety representative(s) under section O.

Attach all appropriate safety documents as specified in this section (e.g., radiation safety protocols, Recombinant DNA documents, etc., in consultation with the appropriate safety officer.

Radionuclides: Identify radioactive isotopes used and their activity. The Radiation Safety Branch's Health Physicists' signature is required.

Biological Agents: List viruses, bacteria, and any blood or body fluids potentially infectious to humans or any human tissue, blood, cells, etc. to be used. A **Human Pathogen Registration Document** (HPRD) must be filed with the Occupational Safety and Health Branch (OSHB) for the use of these biological agents. Identify the HPRD number. For further information consult your IC's Safety Specialist or at the OSHB web site: http://www.nih.gov/od/ors/ds/regprograms.html. (Safety Specialist's signature is required)

Hazardous Chemical or Drugs: List any hazardous chemicals or drugs (carcinogens, mutagens, formaldehyde, inhalant anesthetics, etc.). (Safety Specialist's signature is required)

Recombinant DNA: Identify any nonexempt Recombinant DNA made in the course of this study, i.e. transgenic or knock-out mice. A **recombinant DNA registration document** must be filed with and approved by the IBC prior to initiation of the study. See RDNA Guidelines at: http://www4.od.nih.gov/oba/rac/guidelines/guidelines.html. For further information consult your IC's Safety Specialist. (Safety Specialist's signature is required)

SECTION L: BIOLOGICAL MATERIAL/ANIMAL PRODUCTS

Biological material and animal products such as cell lines, tissues, and tumors have been repeatedly incriminated as vehicles for the introduction of animal pathogens (e.g. ectromelia, lymphocytic choriomeningitis and mouse hepatitis) into NIH animal colonies.

Principal Investigators are responsible for ensuring that the biologic materials used in their study will not endanger the health of the live animals used in their study or other animals housed in the animal facility.

Approval is required prior to introducing any rodent, rodent product or biological material that originate from sources other than those approved by the Veterinary Resources Program. (See NIH Manual 3043-1)

SECTION M: SPECIAL CONCERNS OR REQUIREMENTS

List any items or procedures that may require special care or attention during the performance of the study. Include procedures that may adversely affect the animals, how those effects will be detected and the actions that will be taken to support the animals and to minimize pain or distress.

Information regarding animals that may require special care due to surgical alterations (eg. splenectomy, adrenalectomy, etc.) or genetic manipulations should be recorded.

List any unusual requirements that the animal facility management may need to consider to support the study.

Examples: Importation of animals; specialized housing, lighting, feed, or water; a need for other than routine veterinary care; use and storage of specialized pieces of equipment; special off-hour or weekend/holiday requirements, immunocompromised animals.

Include the justification in this section or an attachment, if nonhuman primates must be exempted from all or part the facility's enrichment plan or if dogs must be exempted from the facility's exercise plan.

SECTION N: PRINCIPAL INVESTIGATOR CERTIFICATIONS

1) Training Course Attendance: If you need the date of your course attendance, or wish to enroll in the next available course, contact your IC Veterinarian's office or the OACU web site at: http://oacu.od.nih.gov .

2) Duplication of Research: As determined by an appropriate literature review.

3) Animal Exposure Surveillance Program (AESP): If information or assistance is needed for enrolling in the Program, contact OMS @ 496-4411 or your IC Veterinarian's office.

4) Animal User Training: Contact the Training Coordinator, Laboratory Animal Care and Use (301-496-5424) for more information.

5) Alternatives: Animal Welfare Act regulations require that "The Principal Investigator has considered alternatives to procedures that may cause more than momentary or

slight pain or distress to the animals, and has provided a written narrative description of the methods and sources..." used to determine that alternatives were not available.

6) Significant Changes: All significant changes must be submitted, in writing, to the ACUC for review and approval **prior to the initiation** of the study change. The ARAC Guideline: "Regarding Significant Changes to ASPs" (3/27/02) defines significant changes as those that have the potential to impact substantially and directly on the health and well being of the experimental animals. The Guideline provides examples of significant changes as: additions or deletions of personnel; addition of surgery; changing animal species, change in pain level, change in overall study objectives, addition of hazardous agents, etc.

For minor ASP changes, refer to your IC's ACUC Chair or Attending Veterinarian for proper reporting procedures.

All ASP forms must be signed by the Principal Investigator.

SECTION O: CONCURRENCES

It is the responsibility of the Principal Investigator to obtain the signature of the Laboratory/Branch Chief.

Proposals originating from a Laboratory/Branch Chief require the concurring signature of the Scientific Director.

The signature of the pertinent OHSB Safety Officer, Facility Veterinarian, etc., must be obtained prior to ASP approval.

SECTION P: FINAL APPROVAL:

The Chairperson of the IC ACUC has authority for final approval of the ASP.

Revised and approved by ARAC, 4/9/2003

Instructions for the "Emergency Animal Treatment & Care Form"

- The NIH intramural IC's will use the standard format provided which will serve as the minimum information to be gathered.
- Additional requested information may be added to this form at the IC's discretion.
- Changes in the format will be considered for approval by OACU.
- Fill out one form for the ASP and submit it along with the completed ASP for ACUC review.
- Multiple forms may be used if care is different for multiple species listed on the ASP or if care is different for individual experimental groups.
- All information listed on this form should be the same as listed in the ASP *without contradiction* regarding animal care issues or endpoints.

- Animal housing location – this is best established prior to completion of the form as the location may dictate the type and level of care that can be provided.
- List of procedures – this should emphasize procedures that may result in serious animal health complications.
- Points of contact (POC) – this does not have to be the PI, but should be the person working directly with the animals who has an intimate knowledge of their experimental status and overall health.
- Potential or expected complications – this should be directly tied to the "list of procedures" and therefore if there are multiple procedures listed, there will likely be multiple complications listed.
- Circumstances requiring contact - this should be directly tied to the "list of procedures" and therefore if there are multiple procedures listed, there will likely be multiple circumstances listed.
- Treatment – if the treatment cannot be prescribed by the veterinarian, then please be diligent in listing restrictions and specific treatments for all listed complications.

Euthanasia – if euthanasia is not at veterinary discretion, then please be diligent in listing restrictions and specific criteria for all listed complications. If the POC must be notified prior to euthanasia, then please ensure accurate contact information and POC availability at critical stages of the experiment.

Instructions for the "Training & Experience Form"

- The NIH intramural IC's will use the standard format provided which will serve as the minimum information to be gathered.
- Additional requested information may be added to this form at the IC's discretion.
- Changes in the format will be considered for approval by OACU.
- Fill a separate form out completely for each animal user listed under Section A of the ASP form, and submit the forms along with the completed ASP.
- The forms are subject to ACUC review.

Administrative info

- Investigator/technician name is the animal user's name.
- PI (Principal Investigator) & AU (Animal User) course completion dates can be obtained from your IC's ACUC coordinator, or if the course was taken on-line, one can re-enter the course and print a certificate that will list one's course completion date.

Training & experience (T&E)

- This should ***only*** address the animal user's T&E for the procedures listed in this ASP and should address both experimental procedures, i.e. injections, blood withdrawals, etc., as well as surgical procedures.
- Short, bulleted statements are sufficient.
- The person responsible for supervision and training does not have to be the PI, but should be the person that has the particular skills and daily interaction to provide training and oversight.

Assurance statements

- Check the two statements as appropriate and have both the animal user and the PI sign the form prior to submission.
- Both statements will need to be verified as "yes" prior to the start of the ASP.

ATTACHMENT 1

Examples of Breeding Schemes

Glossary

Mutant = knock-out or transgenic mouse.
Wild type (+/+) **=** pups from mutant crosses that do not carry the gene of interest at either allele, or background strain that does not carry a mutation.
Heterozygous (het, Aa, or +/-) **=** the mutant gene of interest is carried as one dominant allele and one recessive allele.
Homozygous (hom, AA, or aa) **=** the mutant gene of interest is carried as either dominant or recessive at both alleles.
Background strain = the inbred strain or strains on which the mutant gene was established, e.g. C57BL/6 or 129.
Congenic strains = two strains that are genetically identical except for a short chromosomal segment, achieved by backcrossing to an inbred strain (usually 10 backcrosses).

Maintaining an Established Mouse Strain/Line

When predicting the number of mice required in maintaining an established strain, actual breeding data is used or certain assumptions are made as follows:

- Age of new breeders - both male and female usually mature at 2 months of age.
- Number of pups per litter - 5 pups/litter if average litter size is unknown.
- Number of litters per female - female will produce 4 litters with no postpartum breeding and is typically retired after 6 months.

Example: 2 breeder pairs X 4 litters/each X 5 pups/litter = 40 pups

Mice needed for general experiments

After predicting the number of pups that a breeding pair will produce, it will be necessary to determine how many of the pups will carry the gene of interest and then work backwards to determine the number of breeding pairs needed to support the research. The number of mice needed for an experiment may depend on the phenotype, background strain variability, etc., that is known or thought to occur in the mice. Therefore, it may be useful to include a percentage of all pups, rather than only homozygous mutants (-/-) in the experimental design.

- Recessive or Dominant Mutant (a/a or A/A) maintained via het X het breeding
  Y = number of mutant mice / experiment
  25% of pups will carry gene of interest as (-/-)
  Y/0.25 = total pups needed

  Example:
  Let Y = 100
  100 / 0.25 = 400 pups
  Assume 5 pups/litter X 8 litters/yr = 40 pups/pair/yr
  Therefore: 400 pups/40 = 10 breeder pairs/yr (or 20 breeders total)
  Total mice: 400 pups + 20 breeders = 420 mice

- Recessive or Dominant Mutant (a/a or A/A) maintained via het X hom breeding
  Y = number of mutant mice / experiment
  50% of pups will carry gene of interest as (A/- or a/-)
  Y/0.50 = total pups needed

  Example: Let Y=100
  100 / 0.5 = 200 pups
  Assume 5 pups/litter X 8 litters/yr = 40 pups/pair/yr
  Therefore: 200 pups/40 = 5 breeder pairs/yr (or 10 breeders total)
  Total mice: 200 pups + 10 breeders = 210 mice

Mice needed to generate a congenic line (homogeneous background)

When establishing a new mutant model, it is usually desirable to maintain it on a homogeneous background strain. Many mutants are created on a mixed strain background (usually B6 and 129) that ultimately can interfere with interpretation of experimental results. If the mutant is on a mixed background, a series of backcrosses will both stabilize the allelic position of the mutation (if transgenic) and create a homogeneous background strain. Ideally 10 backcrosses should be performed, which will achieve 99.8% homogeneity. At a minimum, 4 backcrosses should be performed to achieve 94% homogeneity.

Assumption: will maintain two backcross breeding pairs per generation, will produce 5 pups per breeder pair or 10 pups total per generation, and will screen prior to weaning (*if not, then must count +/+ pups in total*).

Breeding scheme:

Donor het x background strain (+/+)	= N1
N1 het X (+/+)	= N2
N2 het X (+/+)	= N3
And so on until N10	

Example:
50% of pups will carry gene of interest as het (+/-)
10 pups X 0.5 = 5 mice / generation
5 mice/generation x 10 generations = **50** donor mice

Need two background strain (+/+) mice / generation
2 x 10 generations = **20** background mice
(Also **2** original donor mice)

Total = 50 + 20 + 2 = 72 mice
Total if screen after weaning: 50 + 20 + 2 + 50(+/+ pups) = 122 mice

Total mice accountability

In addition to the total number of mice needed for an experiment, there must be accountability for the following mice:

- Breeding mice B when mice are bred to maintain a line or produce animals for experiments, some will need to be held back to replenish the breeding pool.
- Cull mice B if pups are genetically screened and culled prior to weaning, they DO NOT have to be counted towards the total number. If genetic screening is performed at or after weaning, ALL MUST be counted toward the total number listed on the protocol.
- Remember, female breeders are usually retired after 6 months.

Example: 2 heterozygous breeder pairs X 4 litters X 5 pups = 40 pups +
4 breeders = 44 mice;

- 10 (-/-) are desired mutants;
- 8 (+/+) used for negative control;
- 2 (+/-) females used to replenish breeding stock at 6 months; and
- 2 (+/+) and 18 (+/-) are euthanized.

ATTACHMENT 2
Guidelines for Pain/Distress Classification

Definitions:
***Pain** is an unpleasant sensory or emotional experience associated with actual or potential tissue damage.[1]
***Distress** is an aversive state in which an animal is unable to adapt completely to stressors and the resulting stress and shows maladaptive behavior.[1]

([1]Recognition and Alleviation of Pain & Distress in Laboratory Animals, National Research Council, 1992)

Column C - Minimal, transient, or no pain or distress
These procedures are considered to produce minimal, transient, or no pain or distress when performed by competent individuals using recognized methods.

1. Administration of:
   a. Anesthetics, analgesics, and tranquilizers
   b. Fluid and electrolyte therapy
   c. Immunizations
   d. Oral medications
2. Peripheral catheterization
3. Blood collection (except intracardiac, and periorbital in some species, see below)
4. Gastric gavage
5. Certain procedures performed in the normal practice of veterinary medicine and those involving the diagnosis and treatment of disease (e.g., injections, palpations, skin scraping, radiography).
6. Euthanasia as performed in accordance with recommendations of the AVMA Panel on Euthanasia.
7. Intracerebral inoculations in neonatal rodents. In many neonatal rodents intracerebral inoculations can be performed by trained personnel prior to cranial ossification, producing only transient pain or distress.

Column D - Pain or distress relieved by appropriate measures
Examples of procedures that may produce pain or distress, but which are performed using appropriate and adequate anesthetics, analgesics, or tranquilizers and followed with appropriate measures to alleviate pain or distress are as follows:

1. All surgery, including biopsy, gonadectomy, and neurophysiological manipulations or preparations such as implantation of electrodes and recording devices, and alterations to nerve or muscle fibers.
2. Burning, freezing, and branding.
3. Fracturing bones.
4. Electrical shocks including shock reinforcement, using voltage that is accepted as generally causing pain in humans.
5. Use of any agent that induces excessive inflammation or necrosis.
6. Drug or radiation toxicity testing, including survivability determinations, producing pain and distress.
7. Chair or stock restraint of un-adapted animals, or of any animal for more than 12 hours.
8. Skin or corneal corrosivity testing, including Draize testing.
9. Intracardiac blood collection.
10. Periorbital collection of blood from any species except mice and hamsters. Note: Periorbital collection from unanesthetized animals that do not possess a true orbital sinus, as do mice and hamsters, is discouraged.

Column E - Unrelieved pain or distress
Procedures, including those listed above for Column D, which are performed without appropriate and adequate anesthesia, analgesia, or tranquilizers; or which are not followed with appropriate measures to alleviate pain or distress; or which are not amenable to relief by therapeutic measures, must be listed in Column E.